AD_____

Award Number: DAMD17-02-1-0615

TITLE: Novel Functional Screen for New Breast Cancer Genes

PRINCIPAL INVESTIGATOR: Mary-Claire King, Ph.D.
Piri L. Welcsh

CONTRACTING ORGANIZATION: University of Washington
Seattle, Washington 98105-6692

REPORT DATE: June 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2004	Annual (1 Jun 2003 - 31 May 2004)

4. TITLE AND SUBTITLE
Novel Functional Screen for New Breast Cancer Genes

5. FUNDING NUMBERS
DAMD17-02-1-0615

6. AUTHOR(S)
Mary-Claire King, Ph.D.
Piri L. Welcsh

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Washington
Seattle, Washington 98105-6692

E-Mail: mcking@u.washington.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Genetic instability is a hallmark of tumor development. Mechanisms for maintenance of genomic stability are heterogeneous and identification of the genes responsible a critical goal of cancer biologists. The very large number of genetic alterations in breast tumors and genetic heterogeneity, even within a single breast tumor, strongly suggests that some mutator mechanism must be involved in breast tumorigenesis. Our hypothesis is that a mutator mechanism contributes to the development of breast cancer. However, since breast tumors do not display an obvious phenotype that signals the presence of a mutator defect (such as microsatellite instability), another scheme to identify defects in repair genes and their targets is necessary. Thus, our first objective is to use a novel yeast model system to identify genes that are previously unrecognized targets of mutator mechanisms and to determine whether these genes are altered in breast tumors. Our second objective is to identify genes that function as novel mutators in the yeast system then evaluate whether any are altered in breast tumors. The identification of mutator genes and their targets that contribute to the etiology of breast cancer will enhance our understanding of the genetic mechanisms involved in initiation and progress of disease. These genes will impact drug and biomarker discovery and ultimately, revolutionize patient care.

14. SUBJECT TERMS
breast cancer, DNA repair, genes, mismatch repair, model systems, mutation, tumorigenesis, yeast

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

All cells are subject to continual DNA damage. For this reason, elaborate pathways have been developed to monitor DNA damage and to coordinate cell cycle progression with DNA repair. To date, over 70 genes involved in DNA damage surveillance and repair have been identified [1]. These genes include those involved in mismatch repair, homologous recombination, non-homologous end joining, and signaling cascades that respond to DNA damage. Of these, only a few (BRCA1, BRCA2, ATM, CHK2, and P53) have been shown to be associated with breast tumor development [2]. However, the very large number of genetic alterations in breast tumors, and genetic heterogeneity even within a single breast tumor, strongly suggest that other, as yet, unidentified repair genes must play a role in breast tumorigenesis.

Our proposal assumed that loss of function mutations in mutator genes contribute to the genetic heterogeneity observed in breast tumors. However, since breast tumors do not display a convenient phenotype (such as microsatellite instability) to signal the presence of repair defects, another scheme to identify mutator genes, and their targets, was necessary. Thus, we designed a straight forward, yeast-based screen to identify these two classes of genes. Our rationale for this project was based on the evolutionary conservation between DNA repair systems in yeast and humans . This conservation enabled us to detect and measure, in yeast strains, increases in mutation rates in human tumor suppressor genes due to defects in known mismatch repair genes [3].

Our research plan consisted of two objectives. First, we proposed to use a novel yeast-based screen to identify genes that are previously unrecognized targets of mutator mechanisms. Second, we proposed to use the same yeast-based screen to identify genes that function novel mutators. Our aims included generating a high quality, complex breast cDNA library, using this library in a screen designed to identify genes that are targets of mutators, screening for novel mutator genes using known tumor suppressor genes as targets, and finally, analyzing both target and mutator genes in high-risk breast cancer families and in sporadic tumors.

Body:

This progress report details all research objectives and progress made to date with an emphasis on progress made during the past year. In addition, we include a comprehensive report of all preliminary data (all data remains preliminary at this time) and research findings for this reporting period (Annual 02).

Statement of Work for DAMD17-02-1-0615 as actually funded.

Technical objective 1: ***Construction of a high quality breast cDNA library***

Months 1-6: Culture normal breast mammary epithelial cells from dissected breast reduction material from premenopausal women. Confirm quality of cultures using immunohistochemical methods. Isolate total RNA, generate poly A+ RNA and convert to cDNA using standard techniques. Clone material into lambda TripLEX. Transduce lambda TripLEX pahgemid library into *E. coli* BNN123 and isolate plasmid DNA.

Studies and Results, June 01, 2002-June 30, 2003

We proposed to construct a high quality normal breast cDNA library using reduction material from 3-4 premenopausal women. Normal mammary epithelial cells have been cultured, the quality of the cultures has been confirmed, and poly A+ RNA has been isolated.

Our rationale for pooling cDNA from different premenopausal women was to normalize the library for genetic contributions that are unique to a particular individual. However, in reviewing our rationale for constructing the breast library solely with cDNA from premenopausal women, we realized that the library should be 'complex' meaning that it should also consist of cDNA from normal epithelial cells from premenopausal women (as proposed), and from postmenopausal women and breast tumors. Thus, construction of such a complex breast cDNA library would allow us to perform a more complete screen.

We have cultured and isolated poly A+ RNA from a number of breast tumor cell lines including those positive and negative for the estrogen receptor. In addition, we have isolated poly A+ RNA from breast tumor cell lines that have no wild-type BRCA1 expression, cell lines that have reduced BRCA1 expression, and cell lines that have wild-type BRCA1 expression. Use of this complex breast cDNA library in our screen will allow for identification of genes that are targets of mutator mechanisms in premenopausal, postmenopausal,and breast tumor cells will be identified.

Technical objectve 2: ***In vivo construction of a breast cDNA library in the yeast vector pCI-HA***

Months 7-8: PCR amplify the normal breast cDNA library with PCR primers to facilitate gap repair. Linearize pCI-HA. Use a high efficiency yeast transformation protocol to transform yeast strains deleted for *msh2* and *mlh1* with linearized plasmid and PCR amplified cDNAs.

Studies and Results, June 01, 2002-June 30, 2003

We originally proposed to construct the breast cDNA library during months 7-8. However, this objective was delayed approximately 4 months to allow for the culture of additional breast cDNAs to make the complex breast cDNA library (see Technical objective 1, Studies and Results, June 01, 2002-June 30, 2003).

Studies and Results, July 1 2003-June 30, 2004

We have completed construction of the complex breast cDNA library. For reasons outlined below, we have not yet used the library in the proposed screen. Thus, there are no preliminary results to present for this objective.

Technical objective 3: ***Screen for targets of mutator mechanisms in the breast transcriptome.***

Months 9-18: Perform dual plating of the normal breast cDNA library in a yeast strain deleted for *msh2* on -Leu/-Ura and -Leu/FOA plates to constrain mutator phenotype. Replica plate clones onto –Leu plates to allow for muation of target sequences. Select for clones that have been disrupted due to defective mismatch repair by replica plating onto either –Leu/FOA or –Leu/-Ura. Repeat the screen in the *mlh1* deletion strain.

Studies and Results, June 01, 2002-June 30, 2003

We have obtained yeast strains defective for *msh2* and *mlh1*. We have confirmed that these strains display a mutator phenotype by transforming them with control plasmids (those containing known targets of these mutator genes) and selecting for mutation events by plating transformants on media containing –Leu/FOA. Mutation rates have been determined and agree with published results. Having confirmed the phenotype of the yeast strains, we are ready to begin the screen to identify targets of these mutators. Because this objective covers months 9-18, we will include progress on this objective in our Annual Report for year 2 of this grant.

Studies and Results, July 1 2003-June 30, 2004

We delayed pursuing this aim over the past year for the following reasons: First, we became aware of two publications that described screens for novel mutator genes [4, 5]. These reports directly impact year three of our proposal. The manuscripts describe the identification of novel mutator genes using approaches that are similar, although not identical, to our approach (See technical objective 6 of this proposal). Because others were already publishing their results, we felt that we needed to begin working on this objective immediately. Hence, most of our research for year two of this proposal (technical objectives 3-5) was postponed until year three.

As reported below, research during year two has focused on technical objective 6. Because preliminary work on this objective proved to be highly successful with, we believe, a greater potential to impact breast cancer, we continue to

pursue this objective. We expect to complete technical objectives 6 and 7 early in year three. Thus, the focus of year three will be the completion of technical objectives 3-4. To our knowledge, no one has performed the type of screen we propose in objectives 3-5. Thus, we felt that delaying objectives 3-5 for a year while pursuing objectives 6 and 7 was worth the risk.

Technical objective 4: ***Identification of target genes and mutations and confirmation of hypermutabilit in candidate genes***

Studies and Results, July 1 2003-June 30, 2004

Months 19-20: Rescue cDNA clones that are disrupted in *msh2* or *mlh1* strains using standard yeast plasmid rescue protocols. Sequence cDNA inserts to identify the gene and mutation. Use candidate cDNA clones to retransform naïve *msh2* and *mlh1*strains and determine mutation rate using data from several fluctuation analyses.

See above for delay on this aim.

Technical objective 5: ***Analysis of candidate genes in sporadic breast tumors***

Months 21-24: Prepare microdissected material from sporadic and inherited breast tumor samples. Isolate DNA from microdissected tumor and normal tissues (normal breast tissue or blood). Sequence candidate genes in tumor and normal DNAs to identify mutations. Repeat procedure for sporadic ovarian tumor samples.

Studies and Results, July 1 2003-June 30, 2004

See above for delay on this aim.

Technical objective 6: ***Screen for novel mutator genes***

Months 25-32: From the haploid set of *Saccharomyces* Genome Project (SGP) deletion strains, remove strains deleted for known mutator genes. Pool remaining clones in subsets. Use high efficiency yeast transformation protocols to introduce plasmid pHJ3. Repeat protocol for plasmids pHJ4 and pHJ9. Identify strains with a mutator phenotype by plating onto –Ura/-Leu to constrain mutator phenotype. Replica plate clones onto –Leu to allow for mutations to occur in target sequences. Select for strains with a mutator phenotype by replica plating onto –Leu/FOA plates. Identify deleted gene in clones which display a mutator phenotype by PCR amplification and sequencing. Determine mutation rates as described.

Studies and Results, July 1 2003-June 30, 2004

We have obtained the complete *Saccharomyces cerevisiae* haploid deletion array collection (BY4741: MATa *his3 eu2 met15 ura3*). We have not removed strains deleted for known mutator genes because we realized that these serve as internal positive controls for our screen. We have generated 7 pools. Pools A-E contain approximately 700 individual clones. Pool F contains about 100 slow growing clones. These clones were pooled independently so as to not bias the collection.

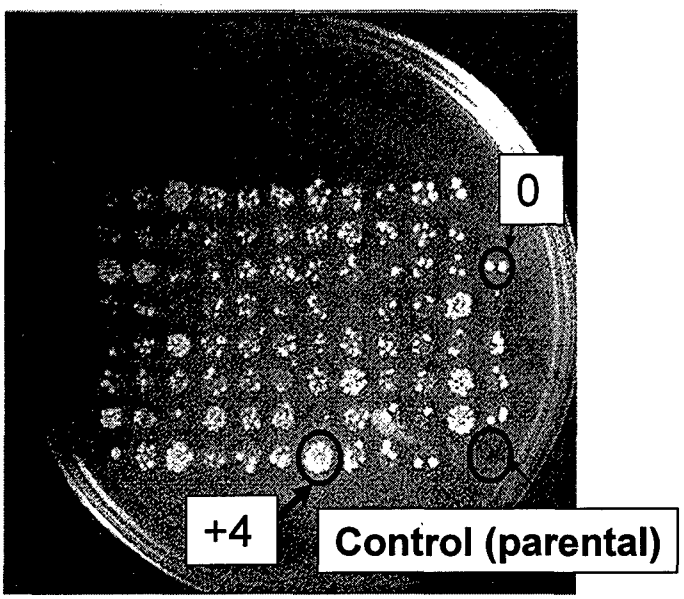

Figure 1

We have optimized our yeast transformation protocol. This modified, high efficiency protocol was used to transform the pools with plasmid pHJ3. Transformed clones were identified by growth on the appropriate selection media. For each pool, 3000 independent transformants were picked and gridded into 96 well plates. Clones were

then replica plated onto media to allow for mutation events to occur. After three days, clones were replica plated onto FOA media to identify FOA^R strains. Strains capable of enhanced growth on FOA due to an acquired mutation in the plasmid target sequence were identified and scored (Figure 1).

Transformants were scored on a scale of 0 to +4 with 0 representing 0-5 colonies per patch, +1 representing 6-12 colonies, +2 represents 13-19 colonies, +3 representing 19-25 colonies, and +4 representing more than 25 colonies or confluent growth. As a control, the parental strain (which does not contain a deletion in any open reading frame) was transformed and carried through the screen (Figure 1). Transformants of the parental strain that grew on FOA media reflect the background mutation rate of the strain. Scoring results for pool A transformants are shown in the following table:

Table 1.

Growth score of strain	0	+1	+2	+3	+4
Number of clones	1412	613	145	92	159
% of total clones screened	59%	25%	6%	4%	6%

In the first round of the screen, 396 strains were scored +2 or greater, with the majority of strains scored as 0. Interestingly, these results are consist with those reported in a similar screen [4]. All 159 clones with a growth score of +4 were re-analyzed on FOA media to determine if the clones continued to display a mutator phenotype. Of these, approximately half (3.6% of all clones analyzed) appeared to be true mutators. Sequence analysis of these clones revealed a number of interesting candidate genes including those involved in signal transduction, sister chromatid segregation, cytokinesis, sumoylation, neddylation, protein synthesis and transport. Candidate mutators are listed in the following table:

Table 2.

Yeast Gene	Function	Human homolog
CYK3	SH3-domain protein with possible role in cytokinesis	SH3KBP1
DCS2	protein of unkown function	DCPS
DDP1	member of the MutT family of nucleotide hydrolases	NUDT10
EFT1	elongation factor2	EEF2
ULA1	involved in protein neddylaiton; plays a role in protein degredation	APPBP1
GAC1	regulates protein phosphatase 1	PPP1RC3
IAH1	iso-amyl acetate-hydrolizing esterase	None
LEM1	multidrug resistance transporter	ABCG2
LEU9	alpha-isoproplmalate synthese II	None
NFI1	SUMO-ligase that interacts with UBC9	PIAS4
PPH21	catalytic subunit of protein phosphatase 2A; regulation of mitosis	PPP2CB
RBS1	RNA-binding suppressor of PAS kinase	KIAA1002
SEY1	protein involved in membrane organization and biogenesis	MYH9
SIA1	suppressor of eIF5A	None
SRC1	protein involved in sister chromatid segregation	LEMD2
STE7	signal transducing MAP kinase kinase activity	MAP2K1
TRS33	a subunit of Trapp, a transport complex involved in ER to Golgi trafficking	TRAPPC6B
YRM1	zinc finger transcription factor; activates genes in multidrug resistance	None
YDL124W	hypothetical open reading frame	AKR1A1
YDL172C	hypothetical open reading frame	None
YOR105W	hypothetical open reading frame	None
YOR111W	hypothetical open reading frame	ASMTL
YOR112W	hypothetical open reading frame	SCYL1
YOR118W	hypothetical open reading frame	None
YOR121C	hypothetical open reading frame	None
YOR129C	component of spindle pole body	None
YOR161C	hypothetical open reading frame	C6orf29
YOR164C	hypothetical open reading frame	C7orf20
YOR166C	hypothetical open reading frame	C1orf26

We are currently in the process of determining mutation rates for all candidate mutators. Those that consistently display a mutation rate above background will be analyzed in sporadic breast tumors as outline in objective 7.

Technical objective 7: ***Analysis of candidate mutator genes in sporadic breast tumors.***

Months 33-36: Use bioinformatic analyses to identify human homologs of novel yeast mutator genes. Prepare microdissected material from sporadic and inherited breast tumor samples. Isolate DNA from microdissected tumor and normal tissues (normal breast tissue or blood). Sequence candidate genes in tumor and normal DNAs to identify mutations. Repeat procedure for sporadic ovarian tumor samples.

Studies and Results, July 1 2003-June 30, 2004

We have identified the human homolog for our candidate mutator genes (Table 2). Additional studies and results for this objective (which covers months 33-36) will be included in the Annual Report for year three of this award.

Key Research Accomplishments:

- We have cultured and confirmed the quality of poly A+ RNA from cultured normal mammary epithelial cells from pre- and postmenopausal women. In addition, we have cultured and confirmed the quality of poly A+ RNA from a variety of breast tumor cells lines

- We have constructed a high quality complex breast cDNA library using poly A+ RNA isolated from normal mammary epithelial cells from pre- and postmenopausal women and from various breast tumor cell lines.

- We have confirmed the mutator phenotype of yeast strains defective in *msh2* and *mlh1*.

- We have screened the haploid set of SGP deletion strains for those that display an elevated rate of mutation. Approximately 4% of all clones analyzed were positive in our screen and thus are candidate mutators. This has been an incredibly successful screen generating a significant number of biologically

interesting candidate genes. These genes will be evaluated in sporadic breast tumors and in high risk breast cancer families to determine if they play a role in development of inherited and/or sporadicdisease.

- We have continued to expand our breast tissue bank. To date, our bank consists of over 400 matched breast tumor and normal tissue samples.

Reportable Outcomes:

We report here the progress of the first and second year of a three-year award. All results remain preliminary thus we have no reportable outcomes. Given that the screen for novel mutator genes will be completed imminently, we expect to submit a manuscript describing this work in the fall of 2004. We also anticipate completion of our screen for novel targets of mutators to be completed early in 2005 and thus expect to submit a manuscript describing that work in the spring of 2005.

Conclusions:

Tumor development is the result of an imbalance between mechanisms controlling gene regulation and genomic stability. Genomic stability is under genetic control. Thus, identification of genes that maintain stability is a goal of cancer biologists. Because a mutator mechanism contributes to the development of breast cancer, we have initiated research designed to identify heretofore, unrecognized targets of mutator mechanisms as well as novel mutators and to determine whether these genes are altered in breast tumors.

The scope of our research includes the evaluation of newly identified genes to determine whether any are altered in breast tumors. Discovery and functional assessment of these genes is essential for understanding the biology of breast cancer and for clinical applications, including identification of therapeutic targets, early breast cancer detection and improved prediction of breast cancer risk and disease course. If we discover novel targets of mutators and/or novel mutators with consequences for breast tumor development, we will open new pathways for investigation into detection and treatment of breast cancer.

References:

1. Wood, R.D., et al., Human DNA repair genes. Science, 2001. **291**(5507): p. 1284-9.

2. Vogelstein, B. and K.W. Kinzler, Cancer genes and the pathways they control. Nat Med, 2004. **10**(8): p. 789-99.

3. Ji, H.P. and M.C. King, A functional assay for mutations in tumor suppressor genes caused by mismatch repair deficiency. Hum Mol Genet, 2001. **10**(24): p. 2737-43.

4. Huang, M.E., et al., A genomewide screen in Saccharomyces cerevisiae for genes that suppress the accumulation of mutations. Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11529-34.

5. Smith, S., et al., Mutator genes for suppression of gross chromosomal rearrangements identified by a genome-wide screening in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A, 2004. **101**(24): p. 9039-44.